ELSEVIER

PII: S0264-410X(97)00073-X

Vaccine, Vol. 15, No. 8, pp. 834–838, 1997

Printed in Great Britain
0264–410X/97 $17+0.00

Protection against tuberculosis by a plasmid DNA vaccine

D.B. Lowrie‡, C.L. Silva†, M.J. Colston* S. Ragno* and R.E. Tascon*

Past attempts to use fractions of mycobacteria as an alternative to BCG have given disappointing results. The availability of cloned genes and suitable vectors has now opened a new avenue in which individual mycobacterial protein antigens are synthesised within transfected mammalian cells. In an ex vivo *transfection approach with a retroviral vector we found that even a single antigen (hsp65) could evoke strong protection when expressed as a transgene and that expression of protection was largely a function of antigen specific cytotoxic T cells. We now find that intramuscular injection of plasmid DNA expressing the antigen from either a viral or a murine promoter can also give protection equivalent to* Bacillus Calmette-Guérin *(BCG). Plasmids expressing some other mycobacterial antigens, hsp70, 36 kDa and 6 kDa, are also effective, suggesting that this approach may lead to a new vaccine. © 1997 Elsevier Science Ltd*

Keywords: tuberculosis, cytotoxicity, IFN-γ

Tuberculosis kills more people each year than any other single infectious agent, about 3 m people. This is despite the existence of highly effective chemotherapeutic drugs and widespread use of *Bacillus Calmette-Guérin* (BCG) vaccine.

Part of the explanation for the modest impact of BCG vaccination on the global problem is that it gives little or no protection in some parts of the world, although it is highly effective in others. Deployment of BCG vaccination also impedes the use of tuberculin skin sensitivity tests as an epidemiological and diagnostic tool to detect tuberculosis and for this reason is not routinely used in the USA. Clearly there is room for a better vaccine.

Major steps forward are now being made as a result of recombinant DNA technology. By expressing mycobacterial genes directly within mammalian cells we have found that individual mycobacterial protein antigens can protect as effectively as the living, antigenically complex, BCG vaccine; the key appears to be the development of an intense antigen specific cytotoxic T cell response following endogenous expression of the antigen as a transgene.

The first stages of this study were done using a retroviral vector to express the *Mycobacterium leprae* gene encoding the 65 kDa heat shock protein (MLhsp65) in the macrophage-like cell line J774. The transfected cells (J774-hsp65) presented the antigen for recognition by major histocompatibility antigen (MHC) class I- and MHC class II-restricted T cells and when they were injected into syngeneic Balb/c mice the mice were protected against subsequent challenge with virulent *Mycobacterium tuberculosis* H37Rv[1]. Allogeneic mice (C57Bl/6), *Listeria monocytogenes* or immunization with the protein with adjuvant all showed no protection. Given the historical background of minimal protection being achieved by immunization with mycobacterial fractions or even with dead bacteria, the high level of protection attained was remarkable.

Limiting dilution analysis[2] of the frequency of hsp65-reactive T cells with $CD4^+CD8^-$ and $CD4^-CD8^+$ phenotypes in spleens of J774-hsp65 vaccinated mice showed that both types were present at very high and equal frequency ($\approx$1:100). Vaccination with live BCG also increased the frequency of both types of hsp65-specific cells equally (to $\approx$1:2500), whereas immunization with the hsp65 protein mixed with J774 cells, or in Freund's incomplete adjuvant, preferentially increased cells with $CD4^+CD8^-$ phenotype. This was consistent with the $CD8^-$ type being particularly associated with protection, an inference that was substantiated by passive transfer of immunity with such T cells.

Protective immunity could be adoptively transferred from J774-hsp65 vaccinated mice to naive mice by hsp65-specific T cells cloned from their spleens[3]. Large numbers of cloned cells (5×10^6) were injected intravenously (i.v.) immediately before the i.v. challenge infection, so the model tested clonal efficacy at the final effector stage of immunity. Clones with $CD4^+CD8^-$, $CD4^-CD8^+$ or γδ-TCR phenotypes were all effective, but the most effective were $CD8^+$.

We have now characterized 12 $CD4^+CD8^-$ and 12 $CD4^-CD8^+$ clones. Specific inhibitors showed that all were conventional in the way that they recognized the antigen presented by macrophages. Proliferation of

*NIMR, The Ridgeway, Mill Hill, London, NW7 1AA, UK. †School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil. ‡To whom correspondence should be addressed.

$CD4^+$ clones in response to exogenous antigen required lysosomal processing, CD4 and matched MHC class II; proliferation and cytotoxicity of $CD8^+$ clones required antigen fragments (produced by tryptic digestion or Golgi processing), CD8 and matched MHC class I. Nine of the 12 $CD4^+$ clones produced interferon-gamma (IFN-γ) and not IL-4; 10 of the 12 $CD8^+$ clones produced IFN-γ, 4 produced IL-4. Seven of the $CD4^+$ clones and 10 of the $CD8^+$ clones were cytotoxic towards macrophages appropriately presenting the antigen; they also lysed syngeneic macrophages when they were infected with *M. tuberculosis* but neither when they were uninfected, nor when the infected macrophages were of different allotype. We conclude from this analysis that the cumulative (secondary) response to J774-hsp65 is predominantly of Th1/Tc1-type with specific cytotoxicity as a major feature.

The T cell clones were able to enhance the antimycobacterial effects of the macrophage: *M. tuberculosis* interaction *in vitro*. Supernatants from only those clones that produced IFN-γ were able to activate macrophages so that when the cells phagocytosed the bacteria the intracellular multiplication was impaired; the effect was prevented by the addition of anti-IFN-γ antibody. Alternatively, if macrophages were first infected then put in direct cell–cell contact with T cell clones, only those clones with cytotoxic activity substantially decreased bacterial growth and this effect was not neutralized by anti-IFN-γ. Thus the clones had two means of enhancing macrophage antimycobacterial activity; one dependent on IFN-γ and one associated with cytotoxicity.

Comparing representative clones for their ability to adoptively convey protection to naive animals (*Figure 1*) it was evident that protection was associated with two properties: a modest effect due to IFN-γ production and neutralizable with anti-IFN-γ; a stronger effect associated with cytotoxicity, particularly with cytotoxicity of $CD8^+$ cells. Hence, a key requirement for a protective effect of vaccination may be that the vaccine sets up the immune response so that a large

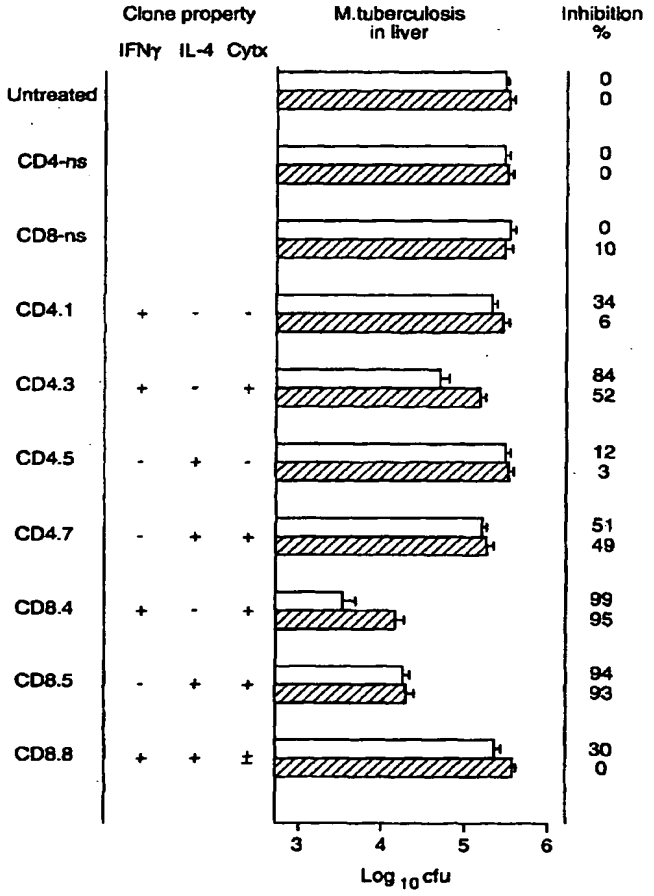

Figure 1 Adoptive transfer of protective immunity against tuberculosis by transfer of T cell clones to naive mice. Mice were γ-irradiated (50 Gy) then injected i.v. with 5×10^6 clone cells and 1×10^5 *M. tuberculosis* H37Rv. In addition, some mice were treated with anti-IFN-γ mAb (0.25 mg/dose) i.p. 2 d before injecting the T cells, immediately before and at 4-d intervals thereafter (shaded bars). The number of live bacteria in the liver was determined as colony-forming units on agar 3 wk after infection. Control mice were either untreated or were irradiated and reconstituted with non-specific splenic T cells enriched from normal mice (CD4-ns, CD8-ns). Percentage inhibition of bacterial numbers was calculated relative to untreated controls. Results shown are means ($\pm$s.d.) from groups of 5 animals.

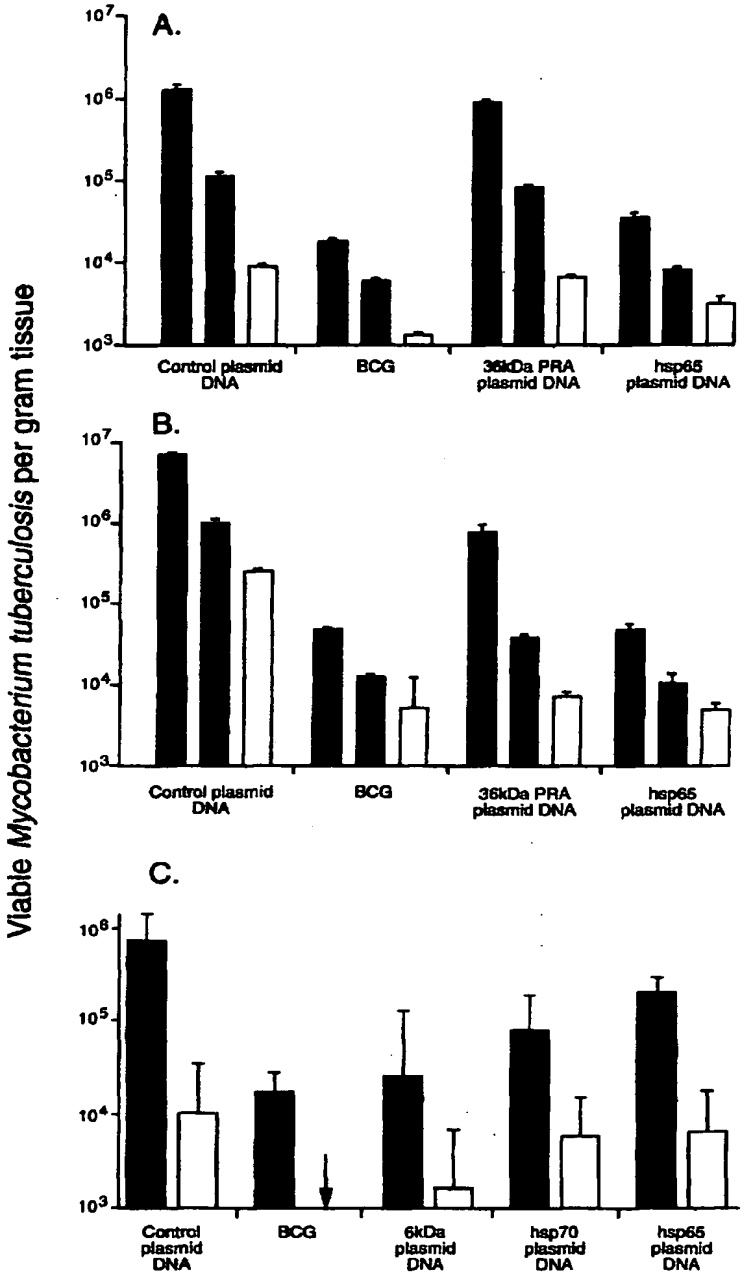

Figure 2 Protection against tuberculosis by immunization with DNA encoding individual mycobacterial antigens. Mice were immunized on four occasions at 3–4 wk intervals by injections of 50 μg plasmid DNA into each quadriceps muscle. (A) Groups of 4 outbred Parkes mice aged 6 wk received into left and right quadriceps muscle respectively either pHMG65 and pCMV3.65, or pHMG36 and pCMV3.36 (encoding 36 kDa proline-rich antigen), or vector without the antigen-encoding sequences, or a single intradermal injection of live BCG vaccine at the time of the first DNA dose. Four weeks after the last DNA dose they were infected by i.p. injection with *M. tuberculosis* and 6 wk later the numbers of live bacteria (mean $\pm$ s.d.) were assessed in spleen (solid bars), liver (stippled bars) and lung (open bars). (B) Groups of 6 crossbred CBA/B10 mice aged 9 wk were immunized with plasmid DNA or BCG and challenged to assess protection as described above, except that pHMG65 was used alone in both quadriceps muscles (50 μg/site). (C) Groups of 4 inbred Balb/c mice aged 5 wk were immunized as above, except that pCMV plasmid contained sequences encoding either hsp65, hsp70 or 6 kDa antigen. The latter was obtained as a 0.3 kb DNA product after PCR amplification of *M. tuberculosis* H37Rv DNA using primers 5'ATTGGATCCGCCATGACA-GAGCAGCAGT3' and 5'TCATTCGAATGCCCTATGCGAACAT3'. After digestion with *Bam*HI and *Bst*BI the product was cloned into vector pcDNA3 and the identity of the cloned product was confirmed by restriction mapping. The hsp70 sequence was a *Hind*III/*Xho*I genomic fragment of *M. tuberculosis* DNA inserted into the pcDNA3 polylinker. Controls were either untreated or received empty vector DNA. Challenge was 3 wk after the last DNA injection and the infection was assessed after 4 wk.

population of antigen specific cytotoxic $CD8^+$ T cells is evoked upon subsequent infection. Vaccination by endogenous antigen arising from a transgene apparently establishes this state, whereas injected antigen (exogenous) does not.

It is now clear that the J774 cell line did not provide, in itself, some unique function for the development of the protective response; bone marrow stem cells transfected using the retroviral vector can also generate protection[4] and so can DNA vaccination. For DNA vaccination we have introduced the hsp65 gene into suitable plasmids downstream of either the cytomegabvirus immediate early gene promoter (pCMV) or the promoter of the murine 'housekeeping' gene for hydroxymethylglutaryl-CoA-reductase (pHMG)[5]. Both promoters effectively drive expression of mycobacterial genes in mammalian cells. In our standard protocol we injected the DNA into the quadriceps muscles of groups of 4–6 mice, 50 μg into the left leg and 50 μg into the right leg, four times at 3–4 wk intervals (400 μg/mouse). Control mice received empty plasmid DNA, hsp65 protein in saline or in Freund's incomplete adjuvant, or a single intradermal dose of BCG at T_0.

Antibody responses were readily detected by ELISA 2 wk after the third dose of plasmid expressing hsp65 and strong lymphoproliferative responses to hsp65 were also found in splenocytes. Cells responding after 4-d culture with the antigen released IFN-γ but no detectable IL-4 (ELISAs), indicating a predominantly Th1/Tc1-type of response. IL-4 (an indicator of a Th2-type response) became detectable *in vitro* after vaccinating with plasmid encoding accessory molecule B7.2[6] in a 50:50 mixture with hsp65 plasmid. The strong Th1/Tc1 bias of the response was further evidenced by RT–PCR[7] analysis of mRNA for cytokines in inguinal lymph nodes draining hsp65 DNA-vaccinated muscle 2 wk after the fourth injection; no mRNA for IL-4, IL-10 or IL-13 was detectable whereas IFN-γ and IL-12 mRNA were increased relative to controls receiving empty plasmid DNA. Splenocytes from hsp65 DNA vaccinated mice also displayed antigen specific cytotoxicity against ^{51}Cr-loaded P815 target cells that had been pulsed with synthetic peptide. Two out of three peptides representing predicted MHC class I restricted T cell epitopes of hsp65 were recognized.

This standard protocol of vaccination with hsp65 DNA protected against tuberculosis challenge (*Figure 2*). The level of protection differed between strains of mice but was high in outbred Parkes mice and equalled the effect of BCG vaccination in CBA/B10 strain. hsp65 is not unique in having this capacity to protect when given as a DNA vaccine. We have now tested 5 known mycobacterial protein antigens individually as DNA vaccines using pCMV constructs in Balb/c mice. Three of them, hsp70, hsp65 and 6 kDa antigen (ESAT-6)[8] elicited significant protection ($P<0.05$; analysis of variance) and two, 36 kDa proline-rich antigen[9] and hsp10, did not (not shown).

We used i.v. and i.p. infections in mice because they are cheap and convenient. Further comparisons in outbred mice and in other experimental models of tuberculosis, such as acrosol infection in guinea pigs, will be needed before conclusions can be drawn as to which antigens are the best candidates for inclusion in a practical vaccine. However, the best antigens may yet remain to be discovered. DNA vaccination lends itself to blind screening of the entire mycobacterial genome for protective antigens (S. Johnston, this issue) and it would be surprising if new candidates are not revealed by this approach.

DNA vaccination may or may not ultimately prove to be a practical alternative to BCG. However, these studies indicate that vaccines that are superior to BCG may not be far away. For example, a vaccine that gives protection equal to BCG by endogenous expression of only a few proteins will leave the majority of the species specific antigens available for diagnostic tests in vaccinated populations. Furthermore, and of potentially far greater significance, there is the prospect that such a vaccine might function in those populations in whom BCG does not.

The reasons for the variable protective efficacy of BCG are not established with certainty. However, it appears likely that sensitization to cross-reactive antigens of environmental mycobacteria prevents the development of a protective response on subsequent BCG vaccination, whereas vaccination of infants before such sensitization occurs gives some protection. If this is so, then a superior vaccine must override such background sensitization. Studies of plasmid DNA vaccination unexpectedly indicate that this may be achievable. We find that vaccination with hsp65 DNA drives a response of an almost purely Th1-type, and generates protection that may depend on antigen specific cellular cytotoxicity (Th1/Tc1-type). E. Raz and his colleagues report elsewhere in this issue that by DNA vaccination a Th1 response can be imposed, and can persist, over the top of a pre-existing Th2-type response. Perhaps vaccination via endogeous antigen can override a non-cytotoxic non-protective response to exogenous environmental mycobacterial antigen and impose a protective cytotoxic response where BCG can not. This hypothesis appears worth testing.

ACKNOWLEDGEMENTS

This work received financial support from the WHO Global Programme for Vaccines and Immunization (GPV), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP).

REFERENCES

1 Silva, C.L. and Lowrie, D.B. A single mycobacterial protein (hsp65) expressed by a transgenic antigen-presenting cell vaccinates mice against tuberculosis. *Immunology* 1994, **82**, 244–248.

2 Taswell, C. Limiting dilution assays for the determination of immunocompetent cell frequencies.I. Data analysis. *J. Immunol.* 1981, **126**, 1614–1619.

3 Silva, C.L., Silva, M.F., Pietro, R.C.L.R. and Lowrie, D.B. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. *Immunology* 1994, **83**, 341–346.

4 Silva, C.L., Pietro, R.C.L.R., Januario, A., Bonato, V.L.D., Lima, V.M.F., DaSilva, M.F. and Lowrie, D.B. Protection against tuberculosis by bone marrow cells expressing mycobacterial hsp65. *Immunology* 1995, **86**, 519–524.

5 Gautier, C., Mehtali, M. and Lathe, R. A ubiquitous mammalian expression vector, pHMG, based on a housekeeping gene promoter. *Nucl. Acids Res.* 1989, **17**, 8389
6 Freeman, G.J., Borriello, F., Hodes, R.J., Reiser, H., Gribben, J.G., Ng, J.W., Kim, J., Goldberg, J.M., Hathcock, K., Laszlo, G., Lombard, L.A., Wang, S., Gray, G.S., Nadler, L.M. and Sharpe, A.H. Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. *J. Exp. Med.* 1993, **178**, 2185–2192.
7 Walker, K.B., Butler, R. and Colston, M.J. Role of Th-1 lymphocytes in the development of protective immunity against *Mycobacterium leprae*. Analysis of lymphocyte function by polymerase chain reaction detection of cytokine messenger RNA. *J. Immunol.* 1992, **148**, 1885–1889.
8 Sorensen, A.L., Nagai, S., Houen, G., Andersen, P. and Andersen, A.B. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infect. Immun.* 1995, **63**, 1710–1717.
9 Thole, J.E., Stabel, L.F., Suykerbuyk, M.E., DeWit, M.Y., Klatser, P.R., Kolk, A.H. and Hartskeerl, R.A. A major immunogenic 36000 molecular weight antigen from *Mycobacterium leprae* contains an immunoreactive region of proline-rich repeats. *Infect. Immun.* 1990, **58**, 80–87.